BASIC METHODS IN

Molecular Biology

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Representations of 24 base pairs of the standard "B" form of DNA, photographed on an Evans and Sutherland PS300 (Arnott, S.; and Hukins, D., Biochem. Biophys. Res. Comm. *47*:1504, 1972). The molecular surface is displayed with dots (Connolly, M. L., Science *221*:709, 1983). Color coding is by atom type: nitrogen is blue; carbon is green; oxygen is red; phosphorus is yellow. The back cover shows the same molecule, cross-sectioned approximately halfway through the helix. Cover illustrations were created by and are courtesy of Dr. J. M. Blaney of the Biomedical Products Department of E. I. du Pont de Nemours and Company, Wilmington, Delaware.

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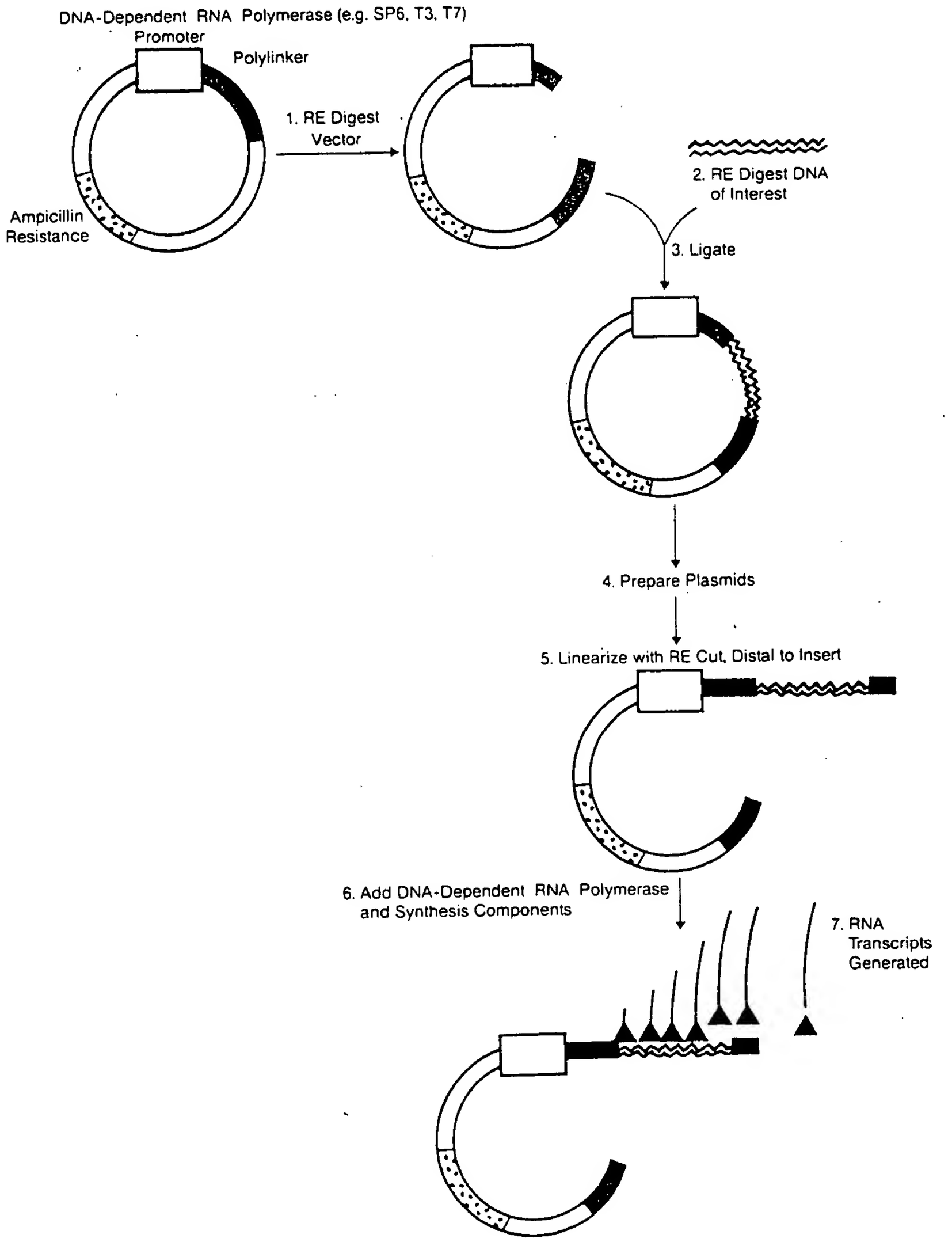
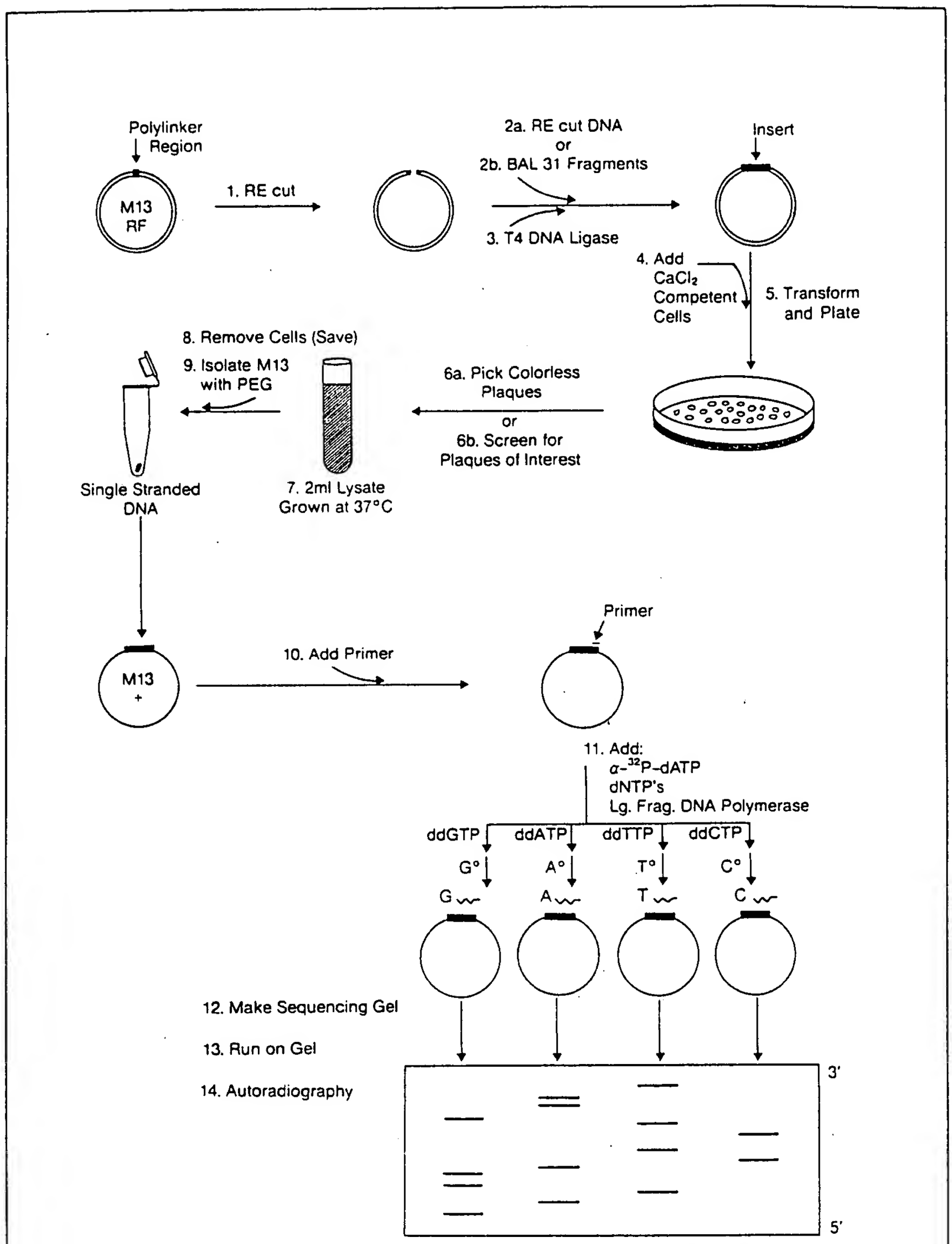


Figure 11.4
Diagram of RNA transcript formation using plasmids with DNA-dependent RNA polymerase promoters, such as SP6, T3 or T7. The DNA of interest is ligated into the vector at compatible RE-cut ends, and plasmids are prepared in the presence of ampicillin for selection. Following linearization with a RE, RNA is transcribed beginning at the promoter region using a DNA-dependent RNA polymerase. (▲=Polymerase, ——— =Newly synthesized RNA strands)



15. Read Sequence From Bottom Up:

5'—G A T G G A C T C T G A A T—3'

STUDIES IN MICROBIOLOGY VOLUME 2

Principles of Gene Manipulation

AN INTRODUCTION TO GENETIC ENGINEERING

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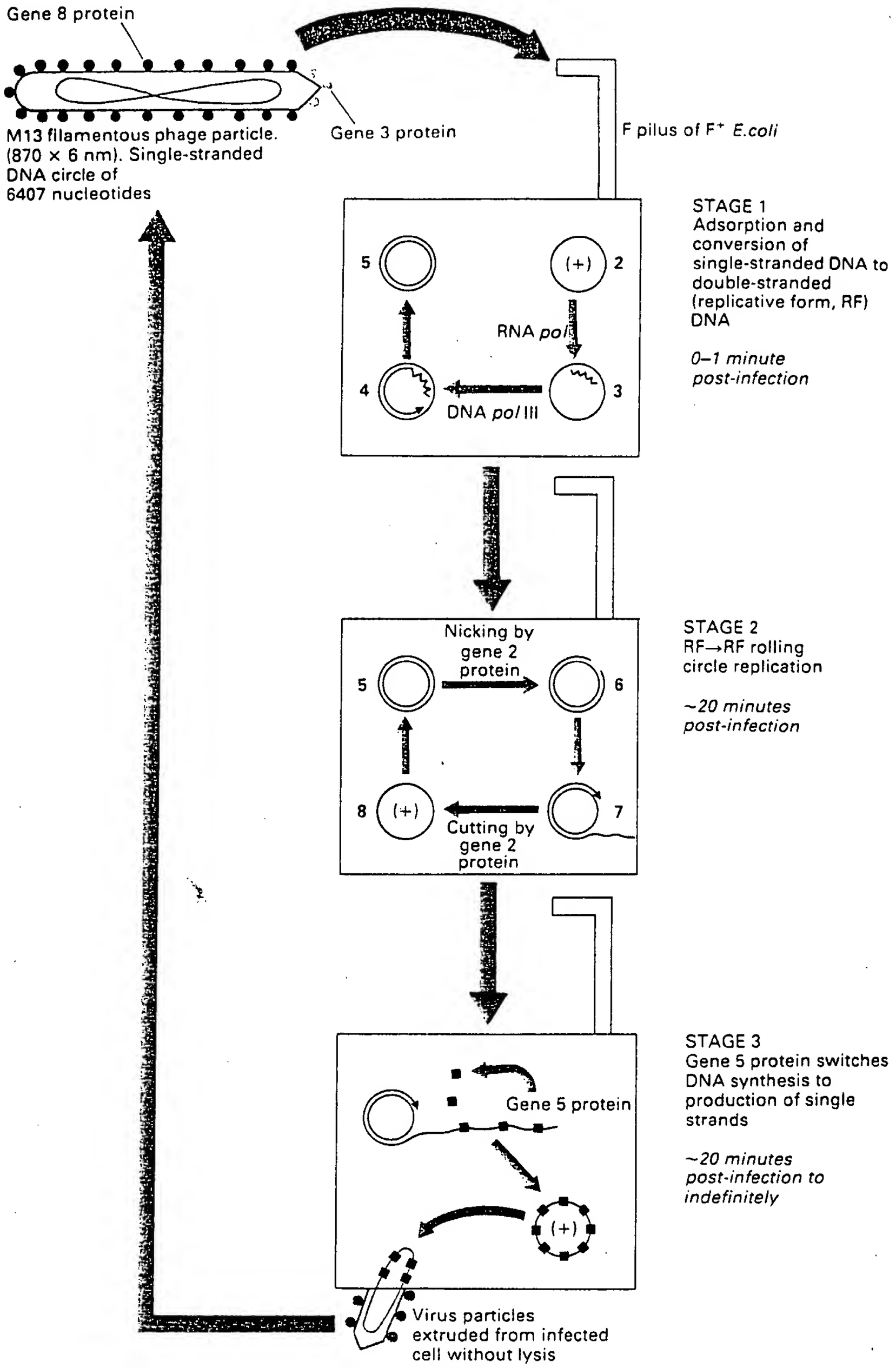


Fig. 4.10 Life cycle and DNA replication of phage M13.

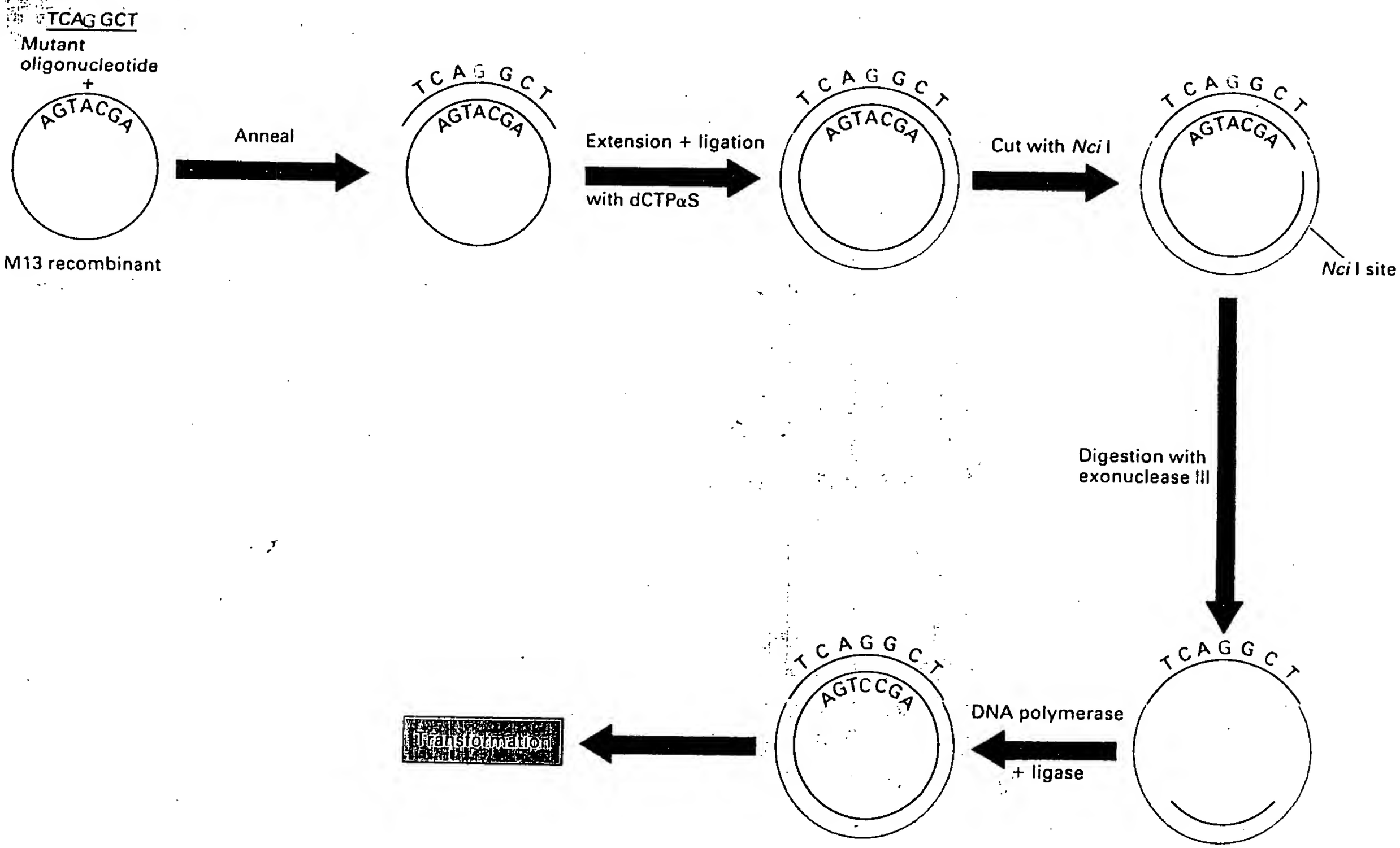


Fig. 5.7 *In-vitro* strand selection of mutants. The DNA-containing sulphur nucleotides is shown in colour.

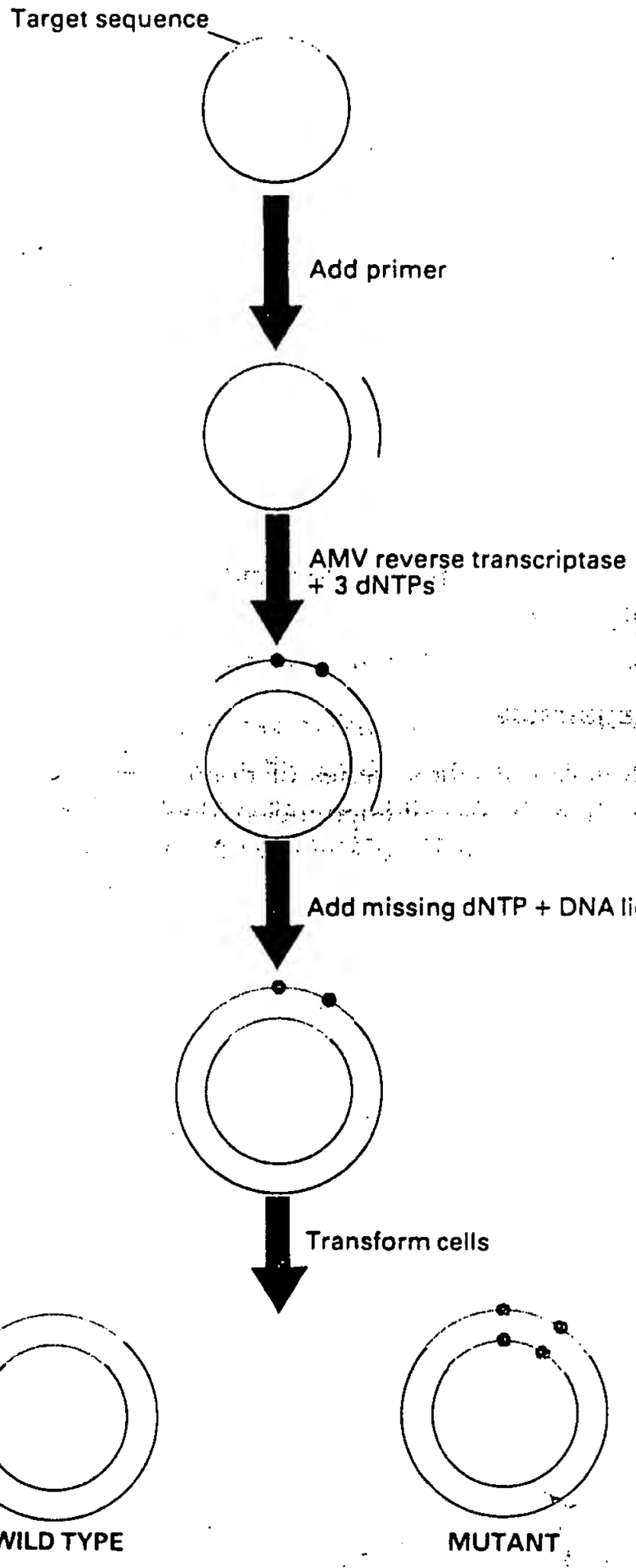


Fig. 5.9 Procedure for random mutagenesis of a target sequence. In the absence of one of the dNTPs the AMV reverse transciptase copies the template DNA slowly and inserts any of the dNTPs present for the missing dNTP. On adding back the missing dNTP, replication occurs at its usual high rate. Dots represent mutated bases.

could correct the *CYC-1* mutation, and revertants were obtained that had the expected base sequence. For this method to succeed it is necessary to have stable mutants and a positive selection method for revertants. High DNA concentrations (100 μg) also are required.

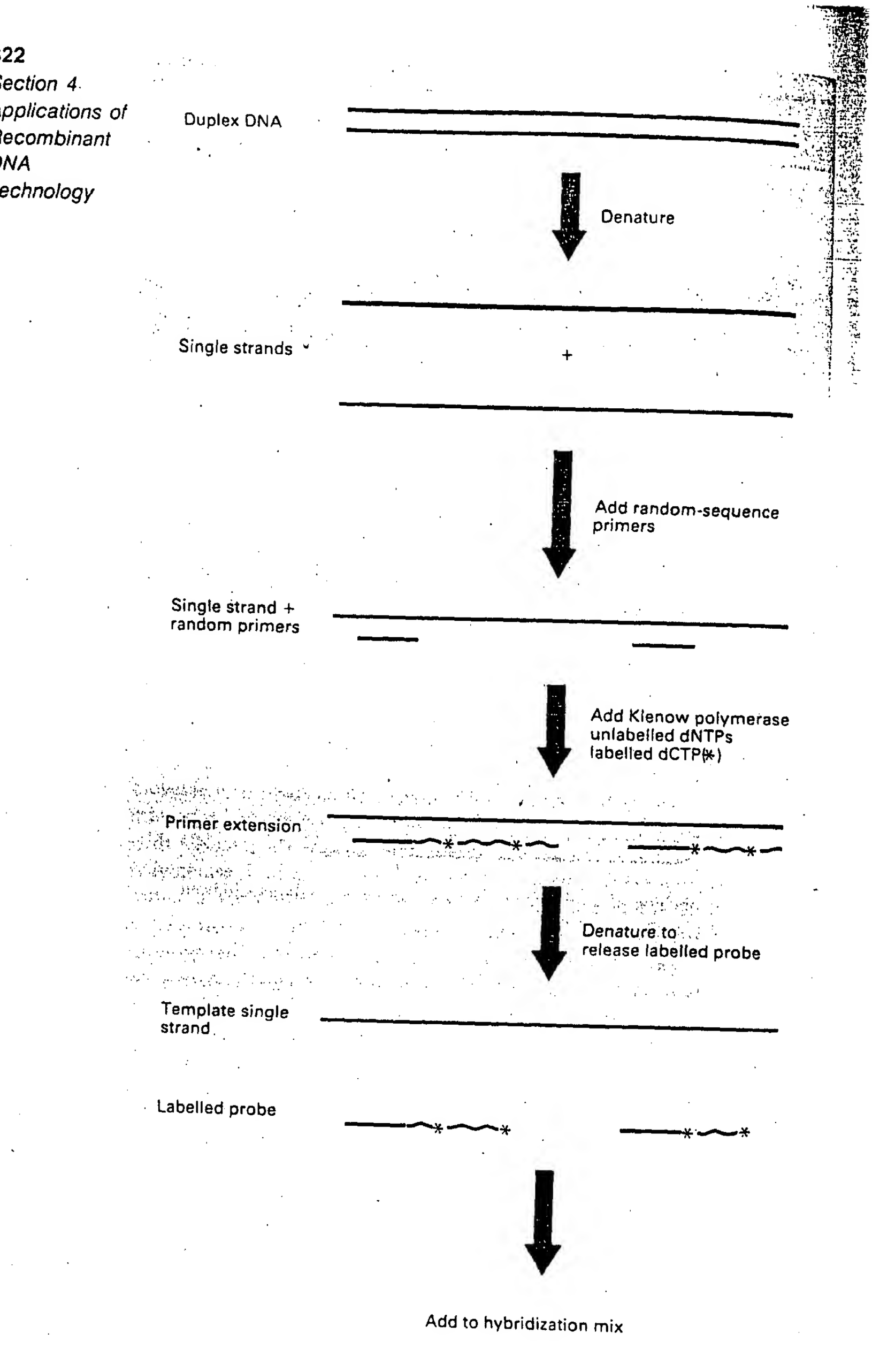


Fig. 15.2 The random primer method for preparing labelled DNA.

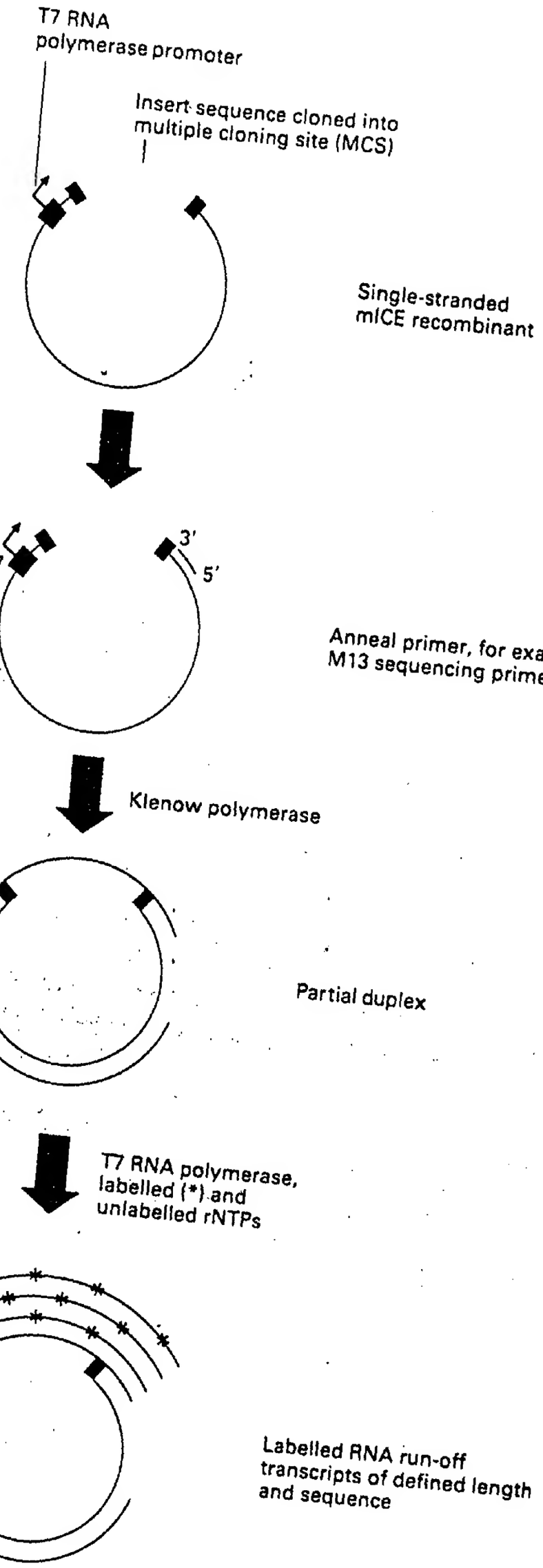


Fig. 15.4 Preparation of probes using a single-stranded mICE vector.

target and the probe nucleic acid are free to move, thus maximizing the chance that complementary sequences will align and bind. Consequently solution hybridizations go to completion five- to 10-fold faster than those on solid supports (Bryan *et al.* 1986). This can be particularly important in many diagnostic microbiology applications where the concentration of the target sequence is very low and speed is essential.

At the end of the hybridization step it is essential to separate duplexes from unbound probe. If one of the two sequences in the hybridization reaction has been immobilized this separation step is achieved by a simple washing procedure. This explains the popularity of filter hybridization, of which there have been numerous examples in previous chapters.

A variation of the filter hybridization reaction is to attach the probe to the bottom of a microtitre plate well or to a tube (Polsky-Cynkin *et al.* 1985). This facilitates the washing step, reduces the total volume of the hybridization, and facilitates the automated reading of results if a colorimetric detection system is used. A clever utilization of this format is the sandwich hybridization reaction (Ranki *et al.* 1983, Palva & Ranki 1985). Here one probe is attached to the solid support and serves to capture homologous nucleic acids. A second DNA probe, which recognizes a contiguous sequence carries the reporter molecule (Fig. 15.12). Although the sandwich format is far less dependent upon the sample composition than direct blotting methods, it is a relatively slow process. Also, the capture is inefficient, since after denaturing the target DNA, the rate of reassociation of the target with itself in solution is considerably greater than its rate of association with the solid-phase probe (Syvanen *et al.* 1986). For the latter reason RNA probes are preferable to DNA probes.

If solution hybridization has been used, removal of unbound probe is not easy. One method is to digest the single-stranded nucleic acid that remains after hybridization with an appropriate nuclease. Although attractive in principle, in practice efficient digestion and separation are difficult to achieve reliably, particularly with crude samples. An alternative method is to separate duplexes from single-stranded by means of differential binding to hydroxyapatite or antibodies specific for double-stranded nucleic acids. A different approach has been adopted by Gingeras *et al.* (1987) who carried out sandwich hybridization in solution. The target DNA is hybridized in solution to a labelled probe and to an unlabelled capture probe which is immobilized by covalent attachment at its 5′ end to a solid support. If the solid support is in the form of beads, subsequent

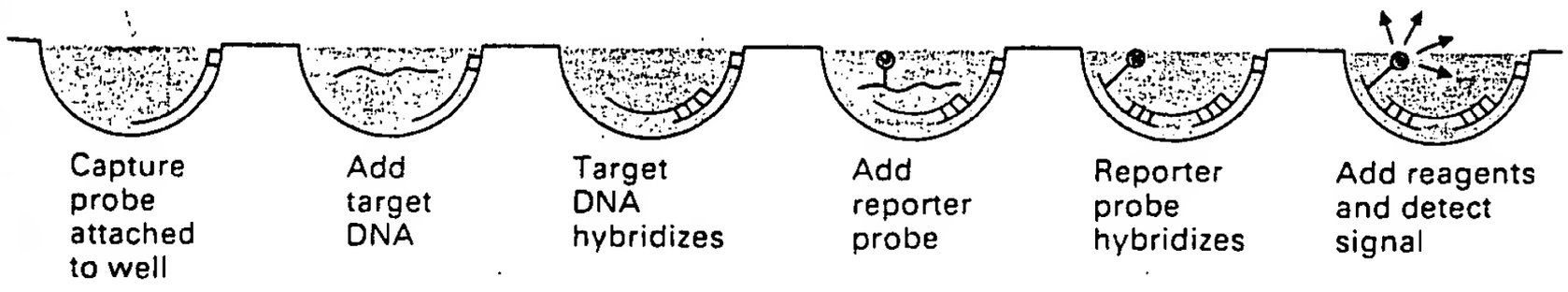
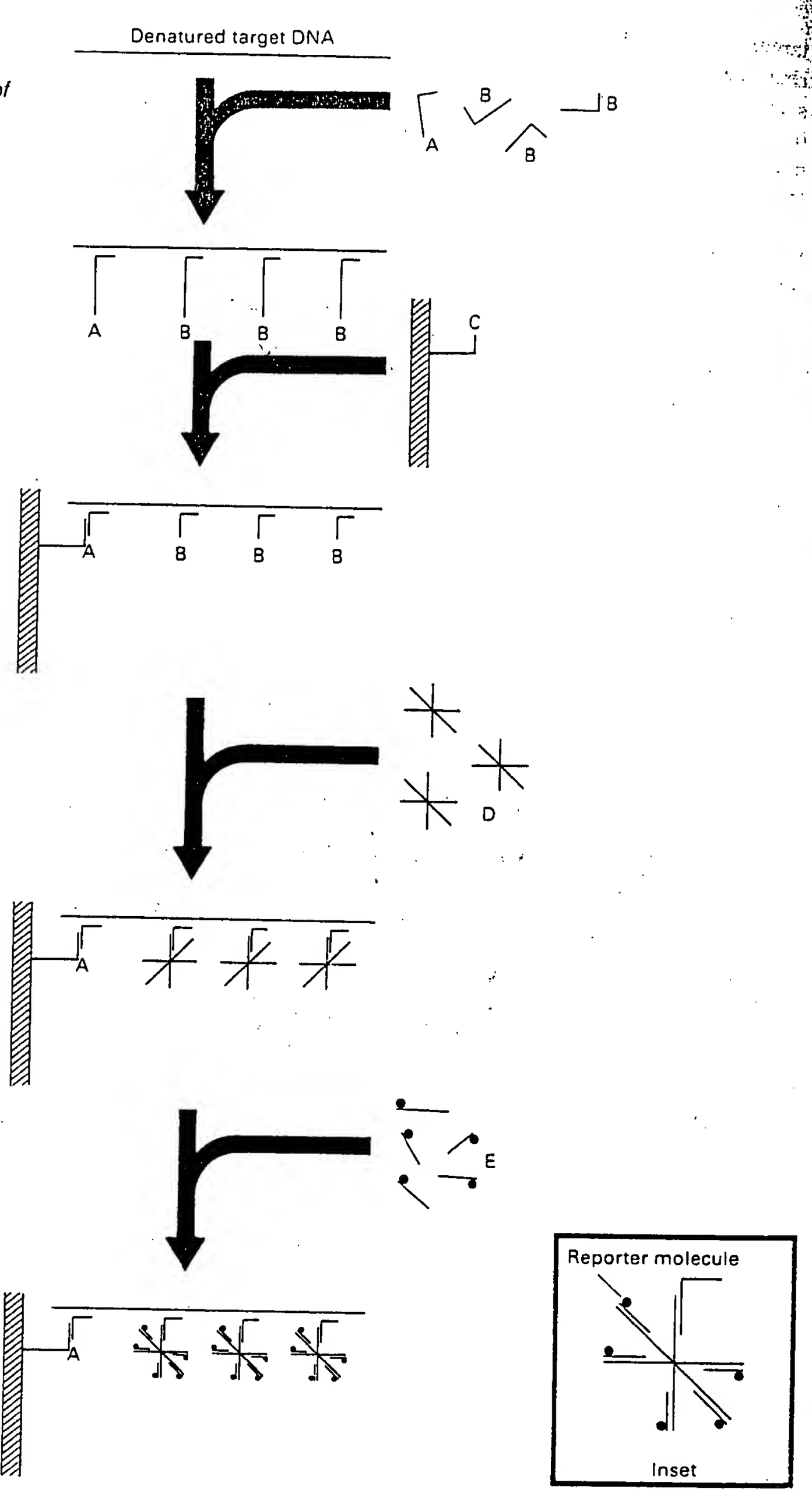


Fig. 15.12 The sandwich hybridization method as carried out in a microtitre tray. The different wells of the microtitre tray show different stages in the procedure.


Denatured target DNA
A
B
B
B
B
A
B
B
B
C
A
B
B
B
D
A
E
A
Reporter molecule
Inset

A PRACTICAL GUIDE TO MOLECULAR CLONING

Bernard Perbal

Institut Curie—Section de Biologie
Centre Universitaire
Orsay Cedex, France

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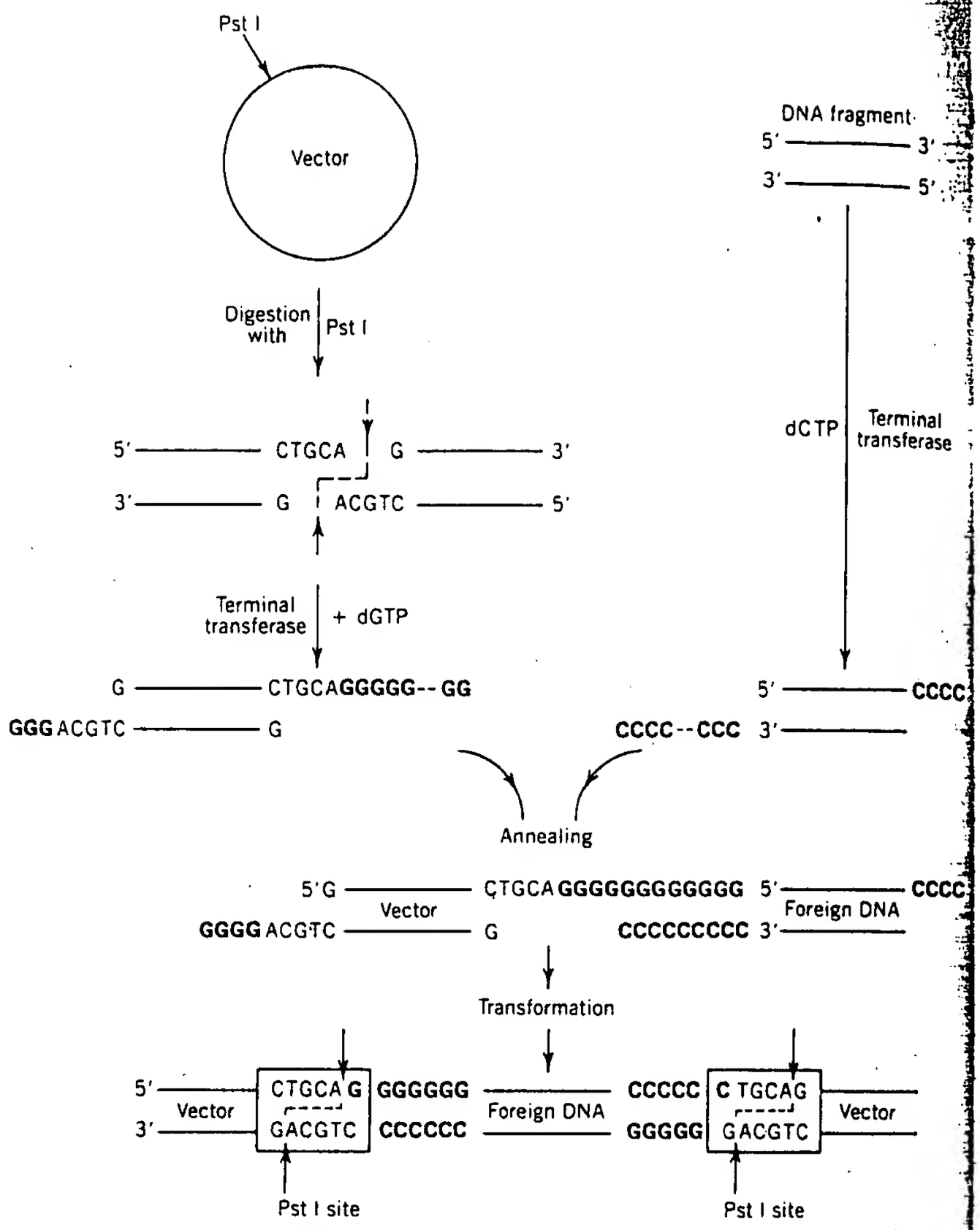


Figure 63. Cloning of a DNA fragment with dC tails into a vector whose cloning site ha been elongated with dG tails. The method shown allows the recovery of the cloned foreig DNA because the Pst I recognition sites are regenerated after ligation.

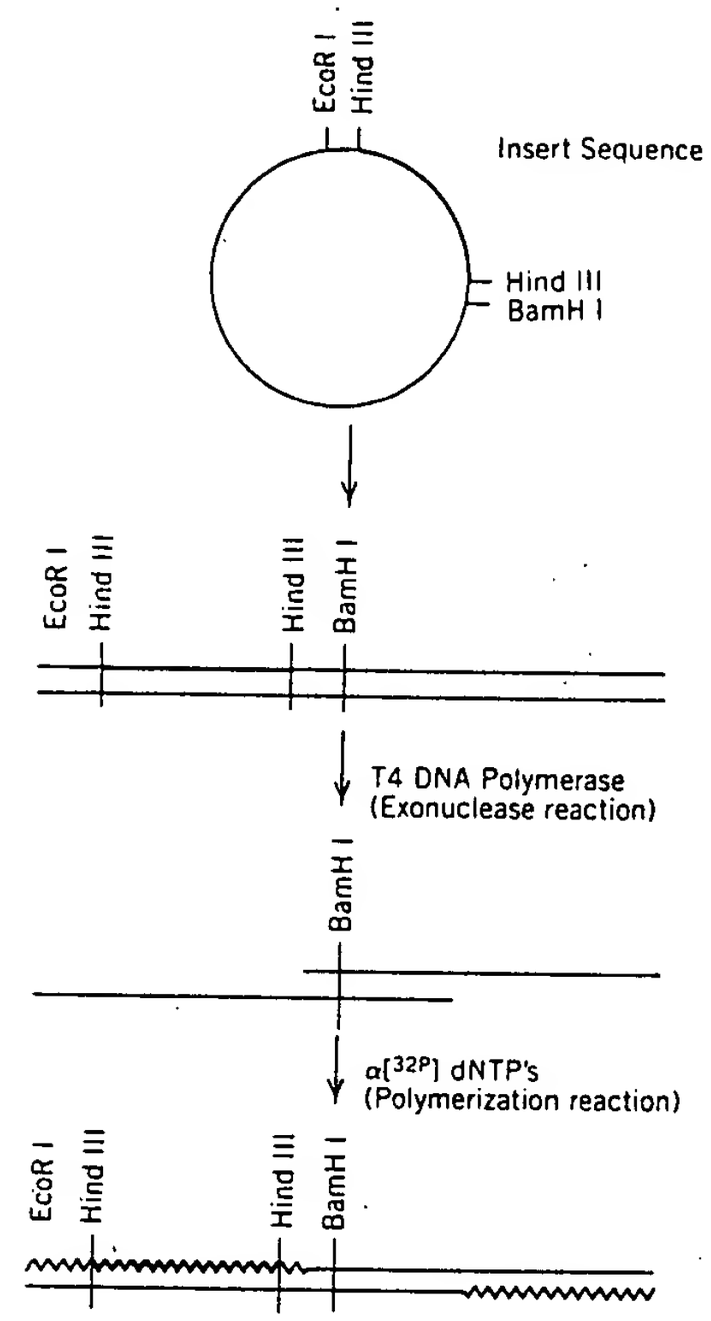


Figure 74. Selective labeling with T4 DNA polymerase of individual strands of a 1-kb insert within the Hind III site of pBR322.

0.5 m*M* DTT

Store as frozen aliquots of a 10× buffer.

Small variations in the buffer composition have no detectable effect on the reactions. The following experimental protocols take advantage of this to simplify the reaction mixtures.

EXAMPLE 1: PREPARATION OF A HYBRIDIZATION PROBE

For this example consider a 5-kb hybrid plasmid with a single Hind III site adjacent to a 1-kb inserted fragment. The object of this protocol is to prepare a hybridization probe for detection of the sequences present on the insert.

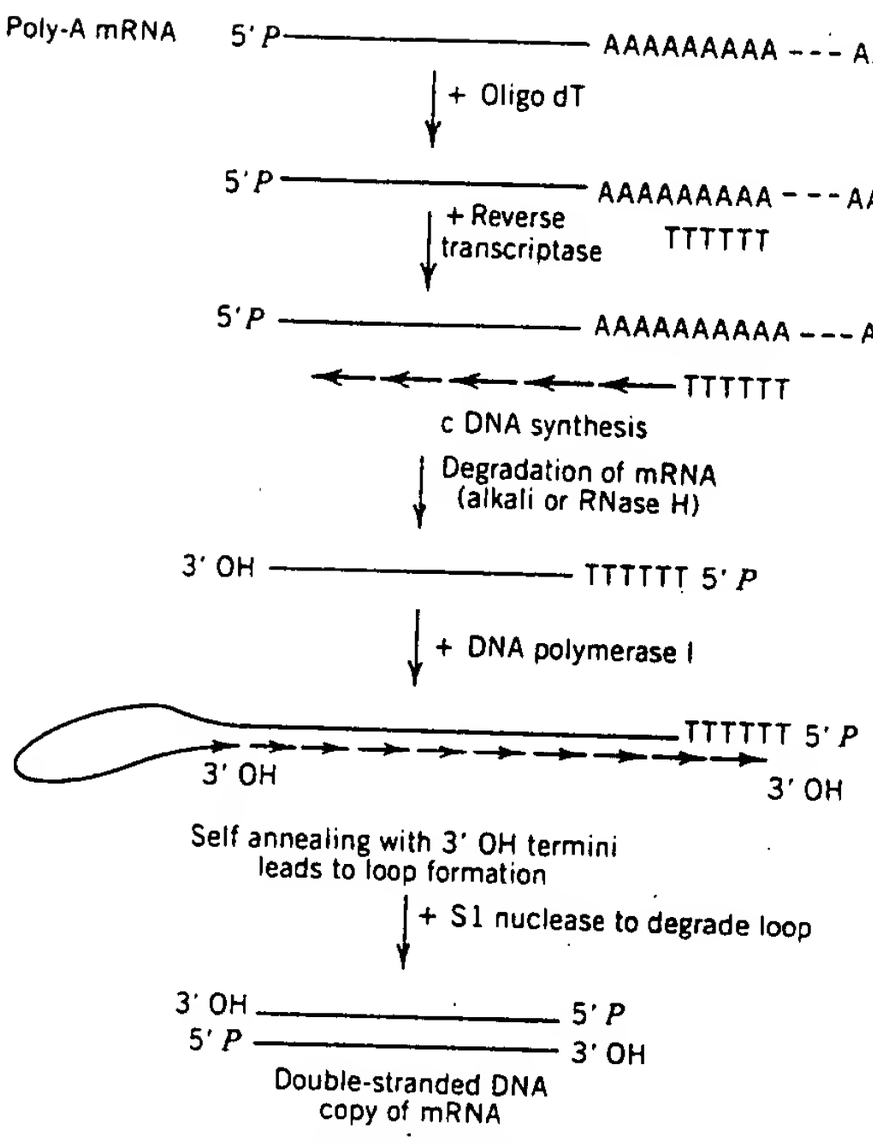


Figure 93. Synthesis of cDNA from poly mRNA.

7. Cool on ice and spin for 2 seconds at 12,000 rpm.

.2. Synthesis of the Second Strand

8. Add:
   - **a.** 5.2 µl sterile distilled water
   - **b.** 2.0 µl 1 *M* HEPES–KOH (pH 6.9)
   - **c.** 1.6 µl 1 *M* KCl
   - **d.** 0.6 µl 0.1 *M* $MgCl_2$
   - **e.** 0.4 µl 0.1 *M* DTT
   - **f.** 0.2 µl 10 m*M* dNTP

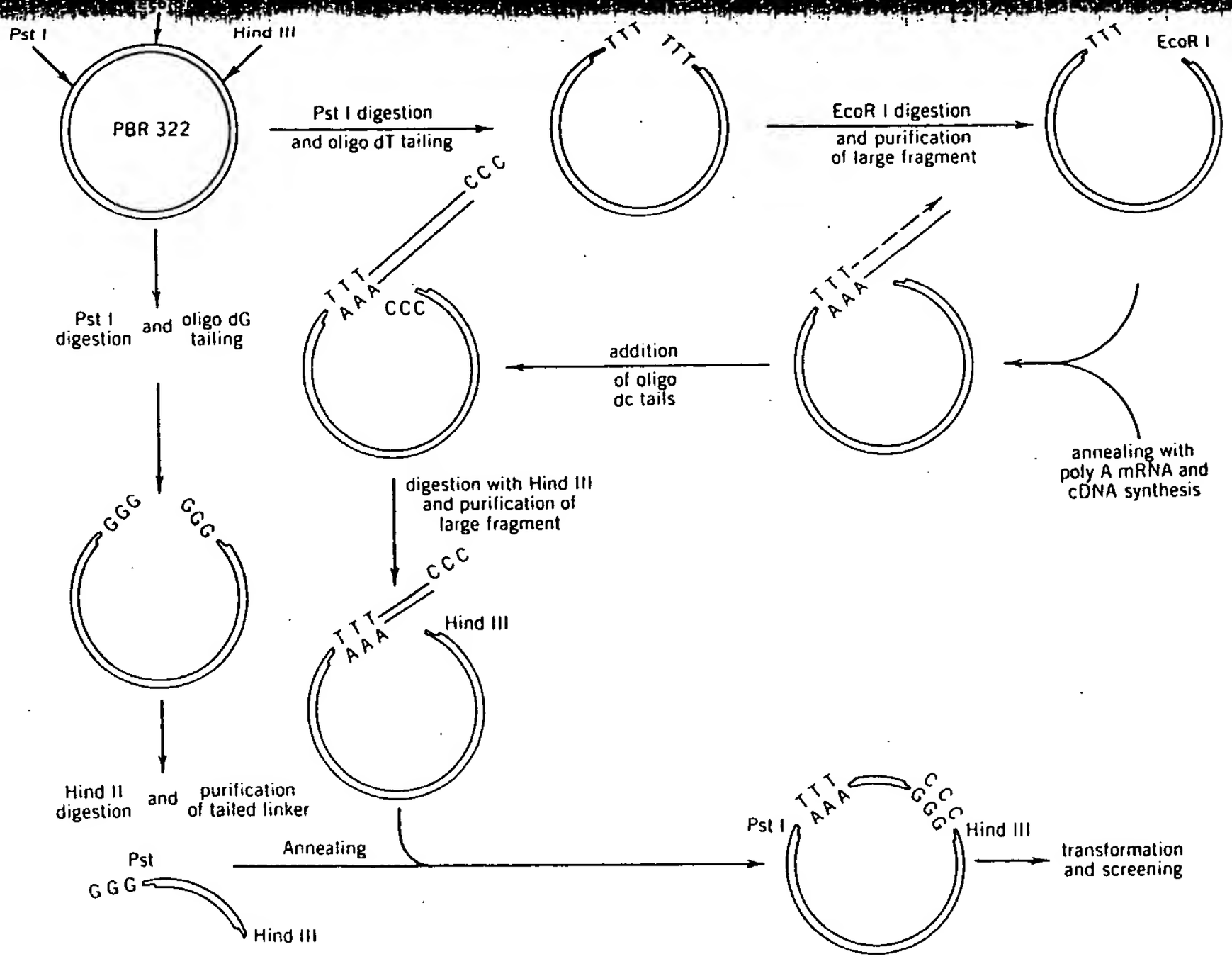


Figure 94. Direct cloning of poly-A RNA species in pBR322. [After Okayama and Berg (1)].

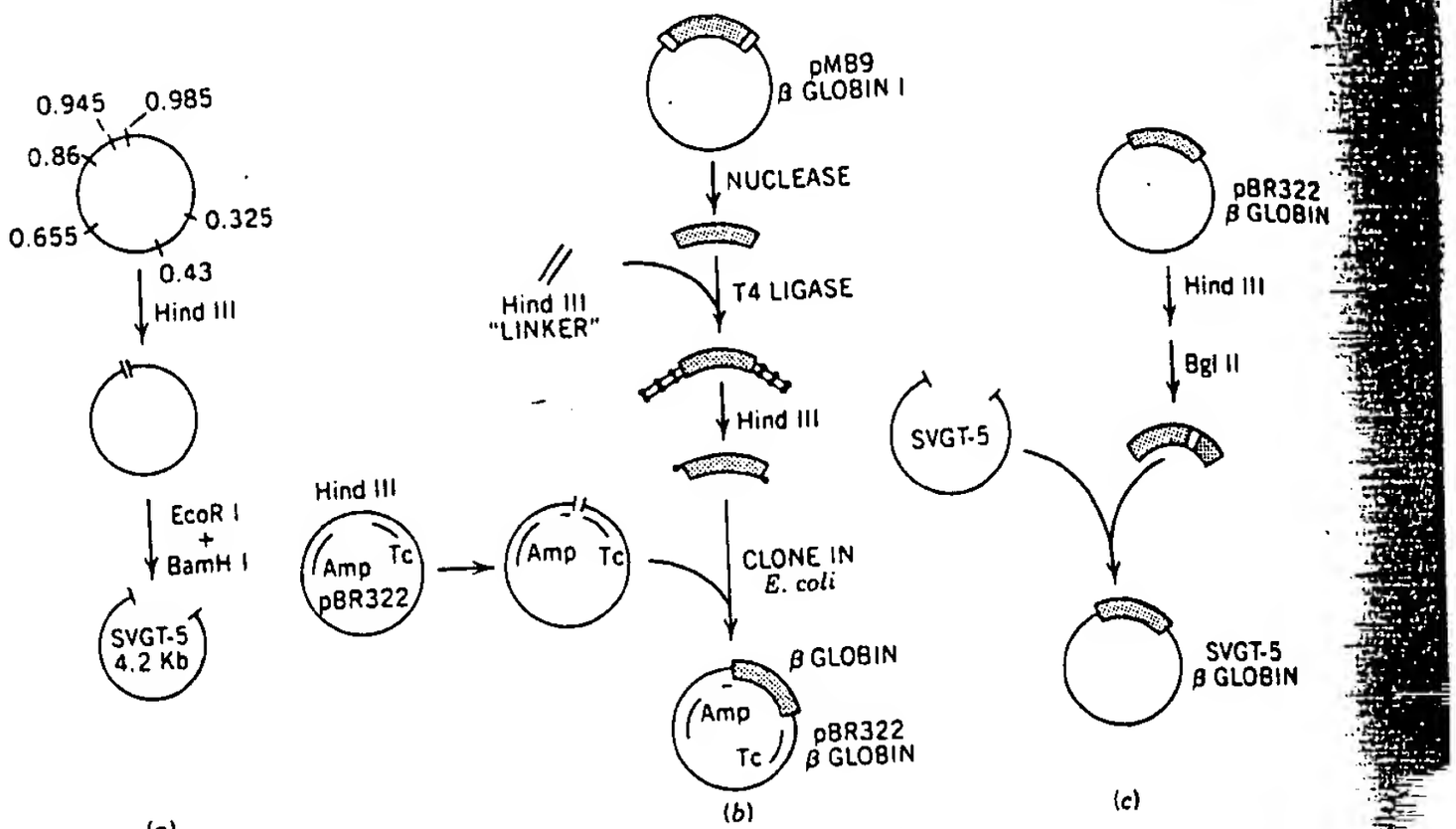


Figure 113. Scheme for construction of the SV40–RaβG recombinant genome. (*a*) Construction of SVGT5 vector. (*b*) Subcloning of β-globin cDNA. (*c*) Cloning of β-globin cDNA into SVGT5. Reprinted by permission from *Nature*, 277, 109. Copyright 1979 Macmillan Journals Limited. [With permission of P. Berg. Copyright (1979) Macmillan Press.]

such amplicons are used to transfect eucaryotic cells (in the presence of helper virus DNA) full-length chimeric defective genomes of approximately 150 kb are generated. They consist of multiple head-to-tail reiterations of the inserted cloned amplicon, which can be introduced back into bacteria, providing a very convenient shuttle cloning system (Figure 118, page 507).

A 12-kb DNA fragment containing the chicken ovalbumin gene with its 5′ and 3′ flanking sequences and an 11.7-kb fragment containing the gene coding for the α subunit of human chorionic gonadotropin have been cloned in the HSV amplicon and stably propagated in virus stocks (Figure 119, page 508).

It is not yet clear if the promoters for eucaryotic genes inserted into defective genomes will be efficiently active in infected cells. However, there is strong evidence suggesting that repeat-unit DNA is preferentially transcribed in infected eells and that more abundant expression of the corresponding gene takes place under these conditions.

REFERENCES

Yeast Vectors

1. See: "Cloning of Genes into Yeast Cells," in *Methods in Enzymology*, Vol. 101, R. Wu, L. Grossman, and K. Moldave, Eds., Academic Press, New York (1983), pp. 167–343.

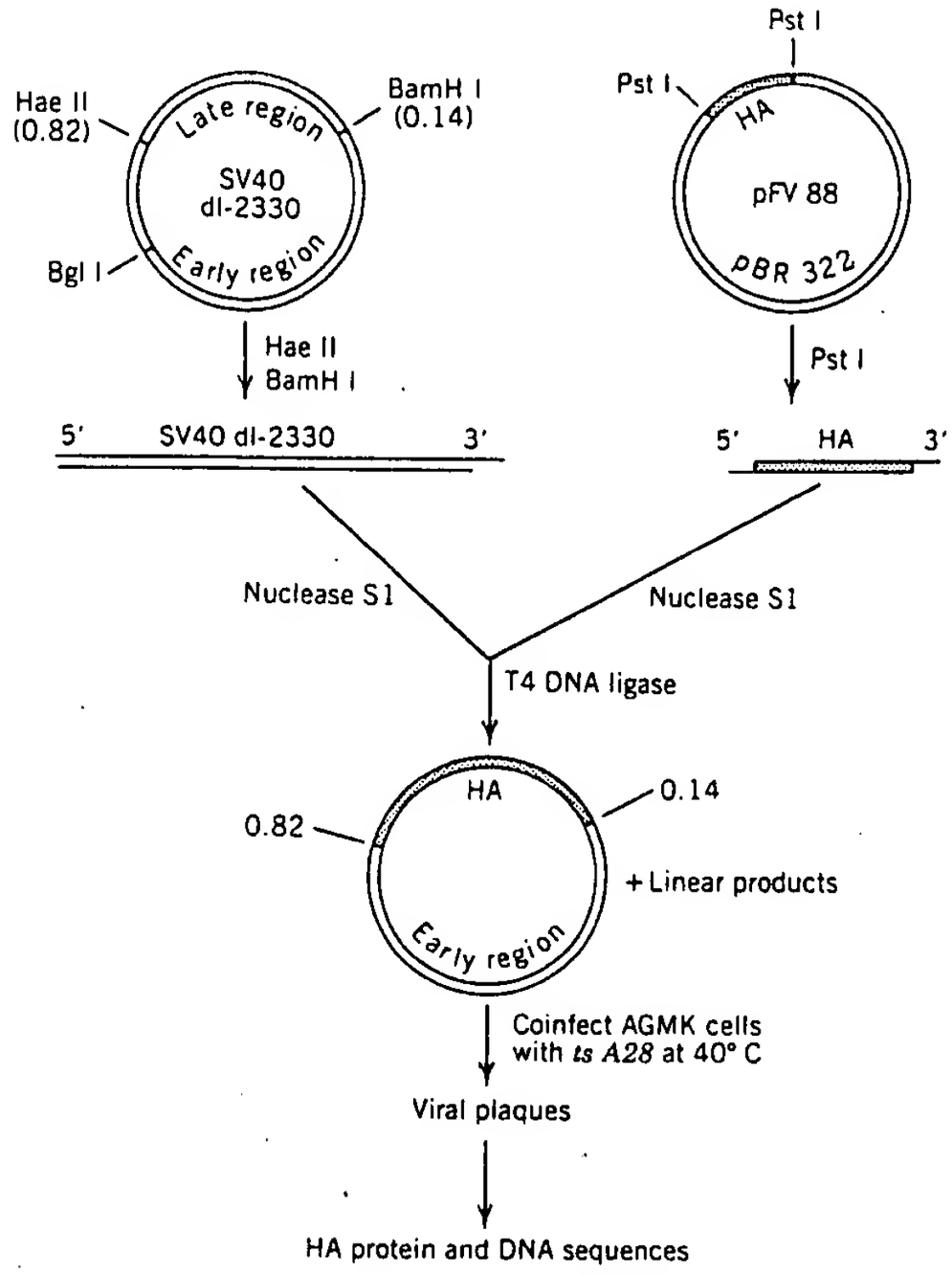


Figure 117. Construction and isolation of a hemagglutinin (HA)–SV40 hybrid virus. (With permission of C. J. Lai.)

19. Stow, N. D., and McMonagle, E. C., in *Eucaryotic Viral Vectors*, Y. Gluzman, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982), p. 199.

For a General Review

20. Gluzman, Y., Ed., *Eucaryotic Viral Vectors*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).

3. TRANSFECTION OF EUCARYOTIC CELLS WITH PURIFIED DNA

Manipulated DNA cannot always be reintroduced into eucaryotic cells by means of nonlytic or nontransforming viral vectors. Techniques have been

Barbara Mansfield
New York
Nov. 1982

Molecular Cloning

A LABORATORY MANUAL

T. Maniatis Harvard University

E. F. Fritsch Michigan State University

J. Sambrook Cold Spring Harbor Laboratory

Cold Spring Harbor Laboratory
1982

Cloning in Plasmids

In principle, cloning in plasmid vectors is very straightforward. The plasmid DNA is cleaved with a restriction endonuclease and joined in vitro to foreign DNA. The resulting recombinant plasmids are then used to transform bacteria. In practice, however, the plasmid vector must be carefully chosen to minimize the effort required to identify and characterize recombinants. The major difficulty is to distinguish between plasmids that contain sequences of foreign DNA and vector DNA molecules that have recircularized without insertion of foreign sequences. Recircularization of the plasmid can be limited to some extent by adjusting the concentrations of the foreign DNA and vector DNA during the ligation reaction. However, a number of procedures, described below, have been developed either to reduce recircularization of the plasmid still further or to distinguish recombinants from nonrecombinants by genetic techniques.

Insertional Inactivation

This method can be used with plasmids that carry two or more antibiotic-resistance markers (see Fig. 1.1). The DNA to be inserted and the purified plasmid DNA are digested with a restriction enzyme that, in this example,

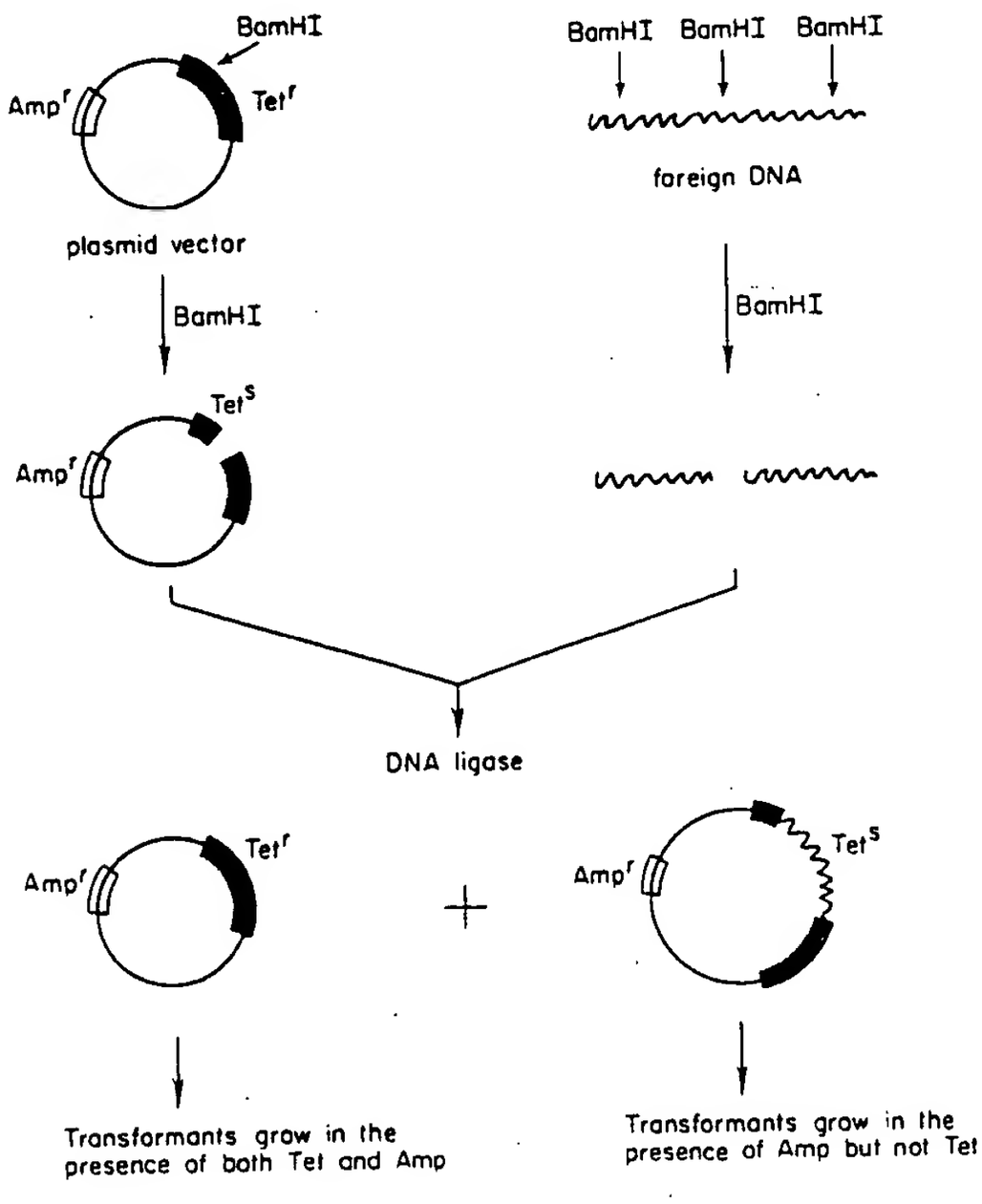


Figure 1.1

Insertional inactivation.

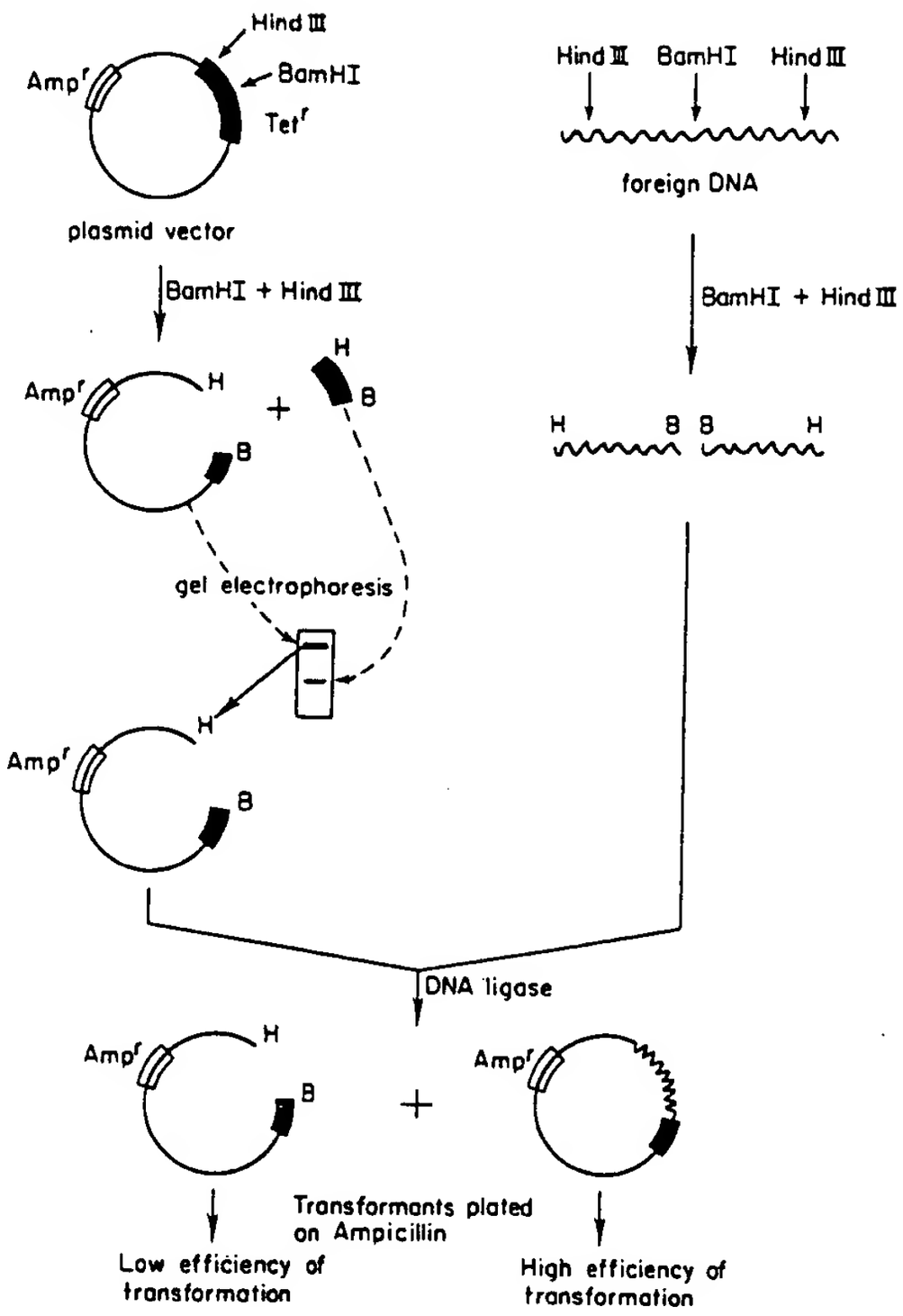


Figure 1.3

Directional cloning.

foreign DNA segment with 5′-terminal phosphates can be ligated efficiently to the dephosphorylated plasmid DNA to give an open circular molecule containing two nicks (see Fig. 1.4). Because circular DNA (even nicked circular DNA) transforms much more efficiently than linear plasmid DNA, most of the transformants will contain recombinant plasmids. A protocol for phosphatase treatment of plasmid DNA is given on page 133.

Problems in Cloning Large DNA Fragments in Plasmids

Finally, the size of the foreign DNA to be inserted can also affect the ratio of transformants containing recombinant plasmids to those containing recircularized vectors. In general, the larger the insertion of foreign DNA, the lower the efficiency of transformation. Thus, when cloning large DNA fragments (>10 kb), it is especially important to take all possible measures to keep the number of recircularized vector molecules to a minimum. Even so, the background is relatively high, and it is usually necessary to use an in situ hybridization procedure (Grunstein and Hogness 1975; Hanahan and Meselson 1980) to identify recombinant transformants.

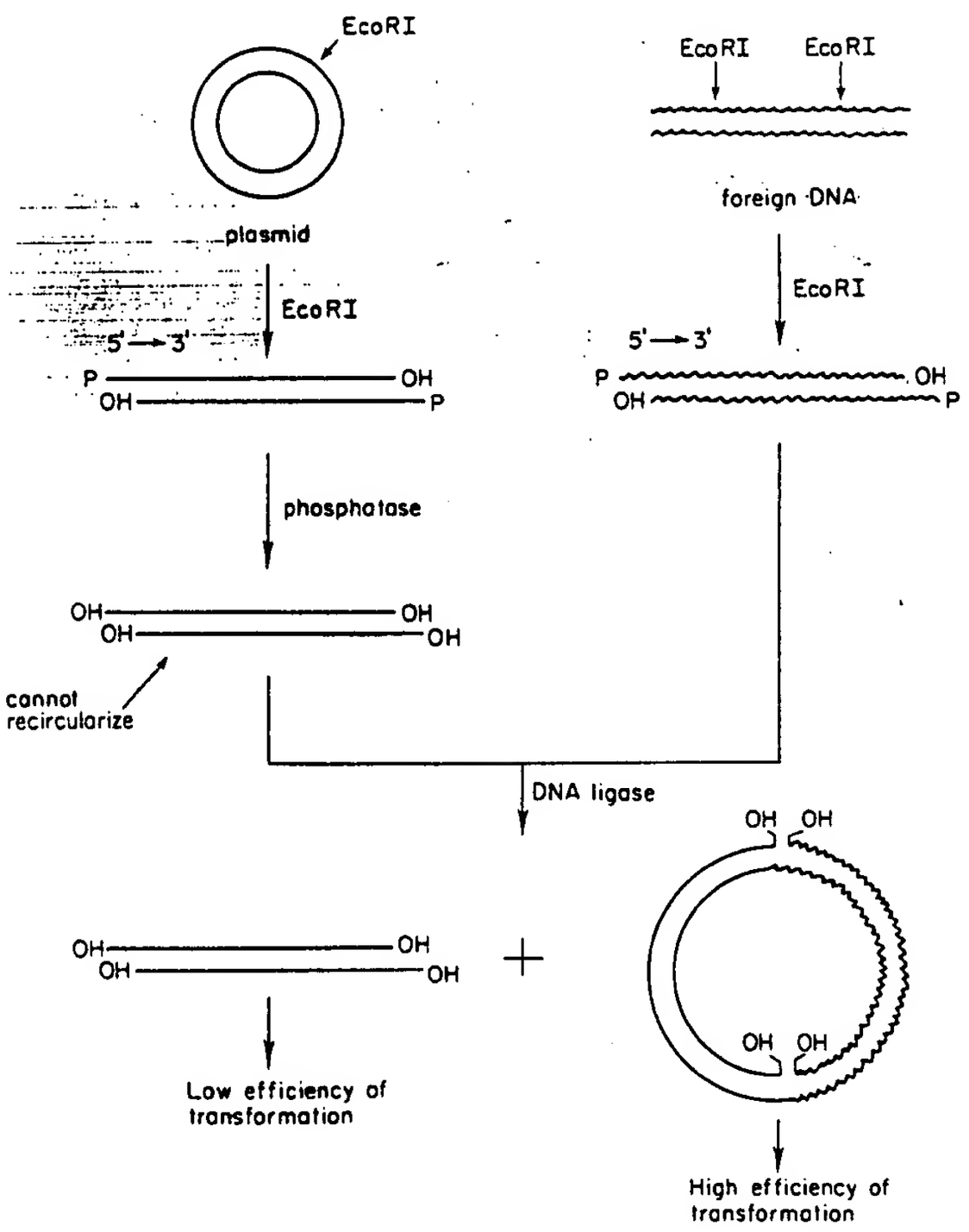


Figure 1.4

Use of phosphatase to prevent recircularization of vector DNA.

The second problem can be minimized by selecting fragments of eukaryotic DNA of a certain size (30–45 kb) for ligation to the cosmid vector. Insertion of two or more such fragments into the same cosmid will result in a molecule too large to be packaged into a bacteriophage λ particle.

Recently, Ish-Horowicz and Burke (1981) have described another method for cosmid cloning that overcomes the first two of these problems. In this procedure, which is designed for the vector pJB8 and illustrated in Figure 1.10, the cosmid vector is divided into two aliquots, each of which is cleaved with a different restriction enzyme cutting either to one side or the other of the *cos* sequence. The resulting full-length, linear DNAs are dephosphorylated with alkaline phosphatase. It is this dephosphorylation that prevents formation of tandem vectors and suppresses the background of bacterial colonies containing cosmids lacking inserts. The dephosphorylated DNAs are then both cleaved with *Bam*HI and mixed to generate cohesive ends, which can be ligated to eukaryotic DNA prepared by partial cleavage with *Mbo*I or *Sau*3A followed by dephosphorylation. This protocol gives only one

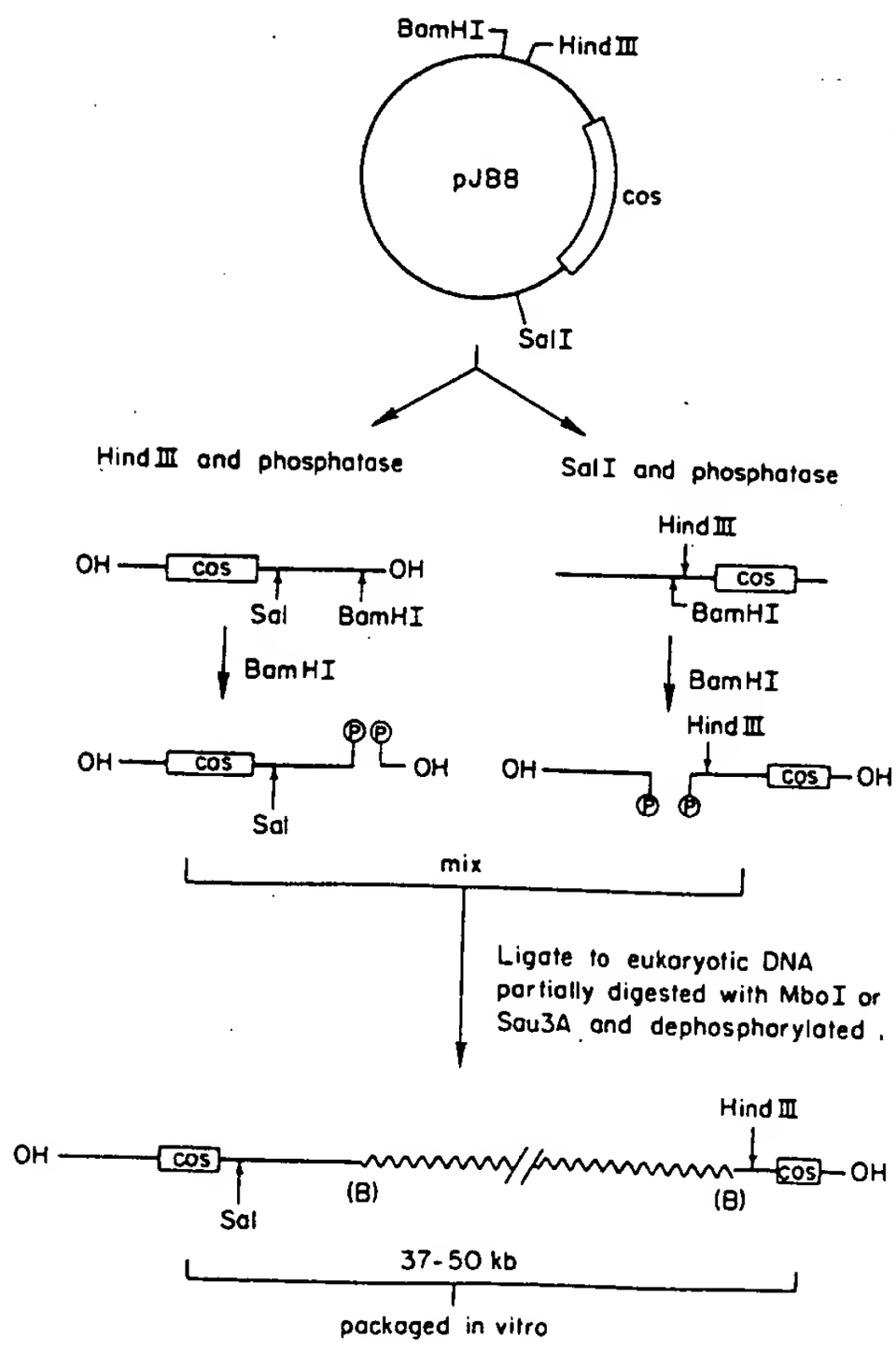


Figure 1.10

Efficient cloning in cosmids (Ish-Horowicz and Burke 1981).

10× nick-translation buffer	5 μl
DNA	1 μg
unlabeled dNTPs (if needed)	1 nmole of each (1 μl of a 1 mM solution)
[α-^{32}P]dNTPs	100 pmoles
H_2O	to 44 μl

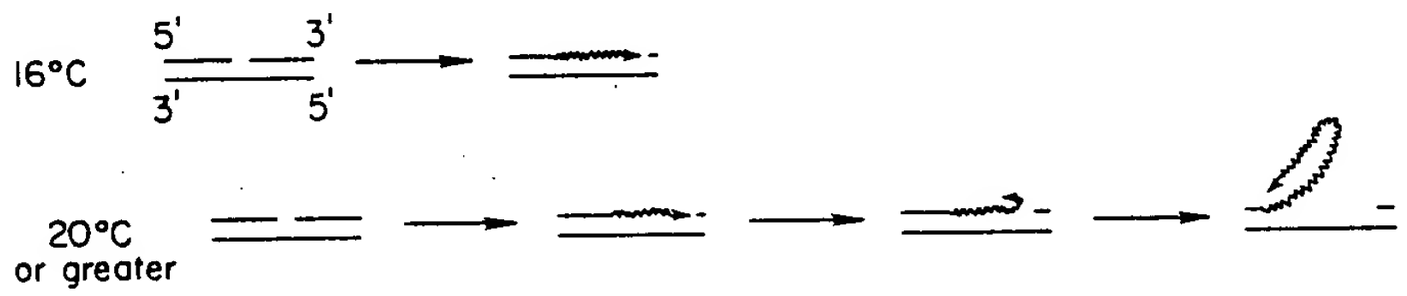
Chill the mixture to 0°C. Make a 10^4-fold dilution of a small quantity of a stock solution of DNase (1 mg/ml) in ice-cold, nick-translation buffer containing 50% glycerol. The diluted enzyme is stable when stored at −20°C in this buffer (see page 451).

5. Add 0.5 μl of diluted DNase I (0.1 μg/ml) to the reaction mixture. Mix by vortexing.

6. Add 5 units (as defined by Richardson et al. 1964) of *E. coli* DNA polymerase I. Mix.

7. Incubate at 16°C for 60 minutes.

   Note. If the reaction is carried out at higher temperature, a considerable amount of "snapback" DNA may be generated by DNA polymerase copying the newly synthesized strand.



9. Stop the reaction by adding 2 μl of 0.5 M EDTA.

10. Using the DE-81 binding or TCA precipitation assays described on page 473, determine the proportion of [α-^{32}P]dNTPs that have been incorporated into DNA.

11. Separate the nick-translated DNA from unincorporated dNTPs either by chromatography on or centrifugation through a small column of Sephadex G-50 (see pages 464–467).

Notes

i. The specific activity of the nick-translated DNA depends not only on the specific activity of the dNTPs, but also on the extent of nucleotide replacement of the template. This can be controlled by varying the amount of DNase I in the reaction. The aim is to establish conditions that will result in incorporation of about 30% of the [α-^{32}P]dNTPs into DNA.

It is possible to obtain more extensive labeling by a replacement reaction, in which the 3′ exonuclease activity of the enzyme first digests duplex DNA to produce molecules with recessed 3′ termini. On subsequent addition of labeled dNTPs, the partially digested DNA molecules serve as primer · templates that are regenerated by the polymerase into intact, double-stranded DNA. Molecules labeled to high specific activity by this technique are used chiefly as hybridization probes. They have two advantages over probes prepared by nick-translation. First, they lack the artifactual hairpin structures that can be produced during nick translation. Second, they can easily be converted into strand-specific probes by cleavage with suitable restriction endonucleases (see Fig. 4.1).

However, this method, by contrast to nick translation, does not produce a uniform distribution of label along the length of the DNA. Furthermore, the 3′ exonuclease activity degrades single-stranded DNA much faster than double-stranded DNA, so that after a molecule has been digested to its midpoint, it will dissociate into two, half-length, single strands that will be rapidly degraded. It is therefore important to stop the exonuclease reaction before the enzyme reaches the center of the molecule. Consequently, the replacement synthesis method yields a population of molecules that are fully labeled at their ends but that contain progressively decreasing quantities of label toward their centers. Thus, the size of the smallest restriction fragment in a mixture of fragments dictates the maximum extent to which all the fragments can be labeled.

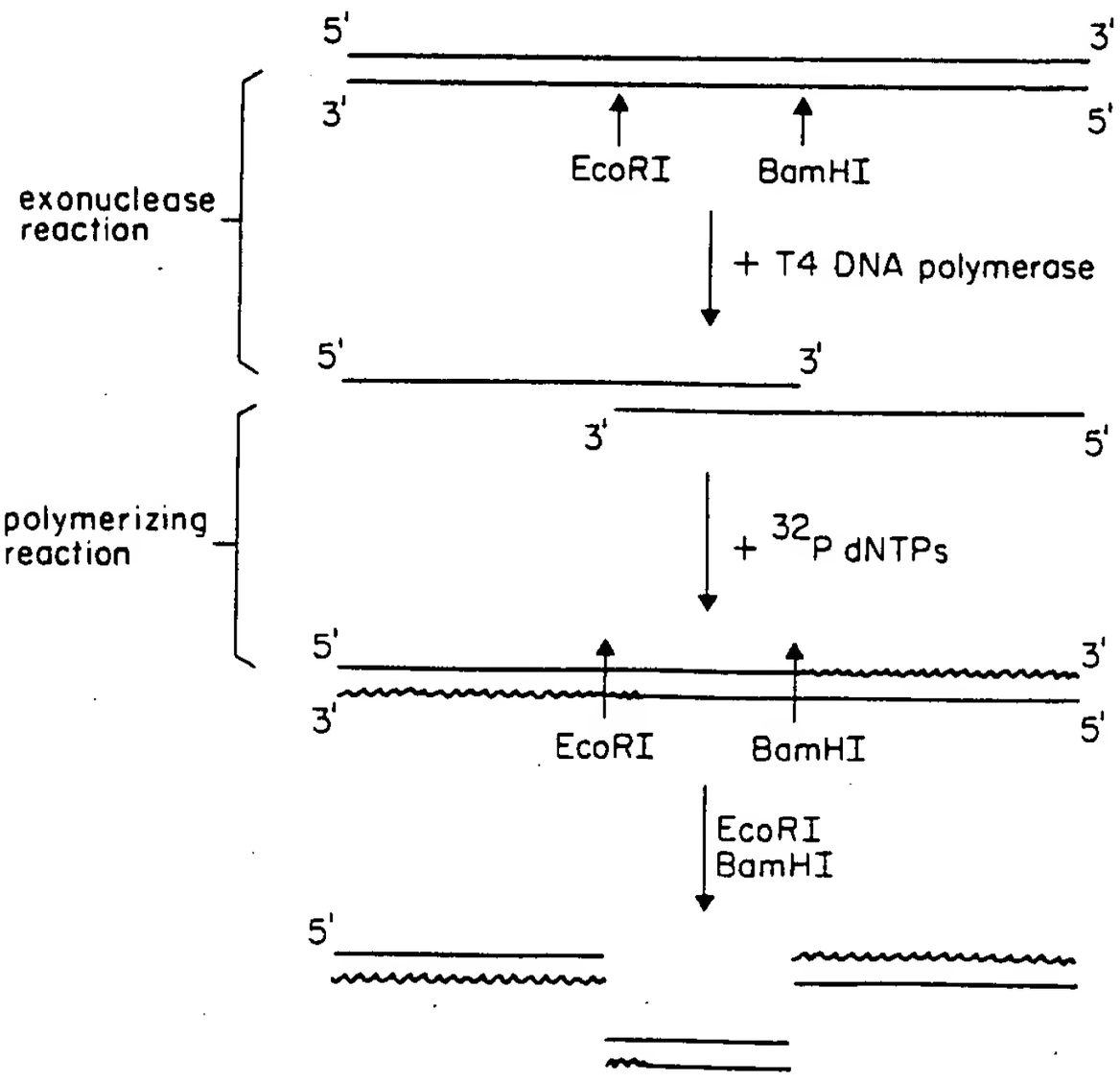


Figure 4.1

Preparation of Hybridization Probes Using Reverse Transcriptase and Random Primers

This is the method of choice for synthesizing ^{32}P-labeled probes of high specific activity from single-stranded DNA or from RNA templates.

1. Mix 1.0 μg of template (linear double-stranded or single-stranded DNA or RNA) with 20 μl of water. Heat to 100°C in a boiling-water bath for 5 minutes. Chill quickly in ice water.

2. Add:

calf thymus or salmon sperm primer (50 mg/ml)	10 μl
5× random-primer buffer	20 μl
2 mM solution of each unlabeled dNTP	2 μl
[α-^{32}P]dNTP (sp. act. > 400 Ci/mM)	250 pmoles (100 μCi)
reverse transcriptase	200 units
H_2O	to 100 μl

5× Random-primer buffer

0.25 M Tris · Cl (pH 8.1)
10 mM dithiothreitol
25 mM $MgCl_2$
0.2 M KCl

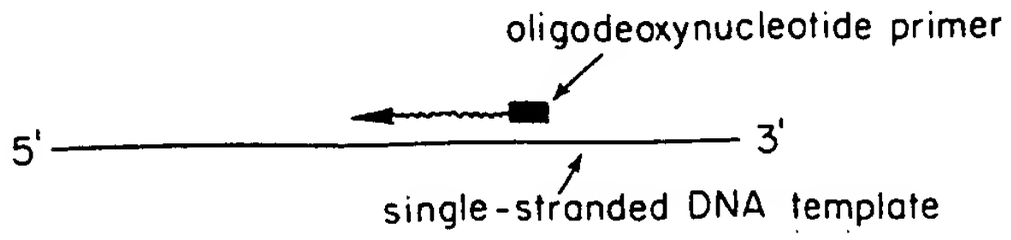
3. Mix and incubate at 37°C for 1 hour.

4. Add 2 μl of 0.5 M EDTA. Separate the labeled DNA from unincorporated dNTPs either by chromatography through a column of Sephadex G-50 or by spun-column chromatography (see pages 464–467). Approximately 30% of the [α-^{32}P]dNTP should have been incorporated into DNA.

Notes

i. RNA templates can be removed at the end of the reaction as follows: After addition of EDTA (step 4), add 12 μl of 3 M NaOH and incubate for 12 hours at 37°C. The alkaline solution can then be applied directly to Sephadex G-50 equilibrated in TE (pH 7.6).

ii. Hybridization probes made by reverse transcriptase from DNA are single-stranded copies of the templates:



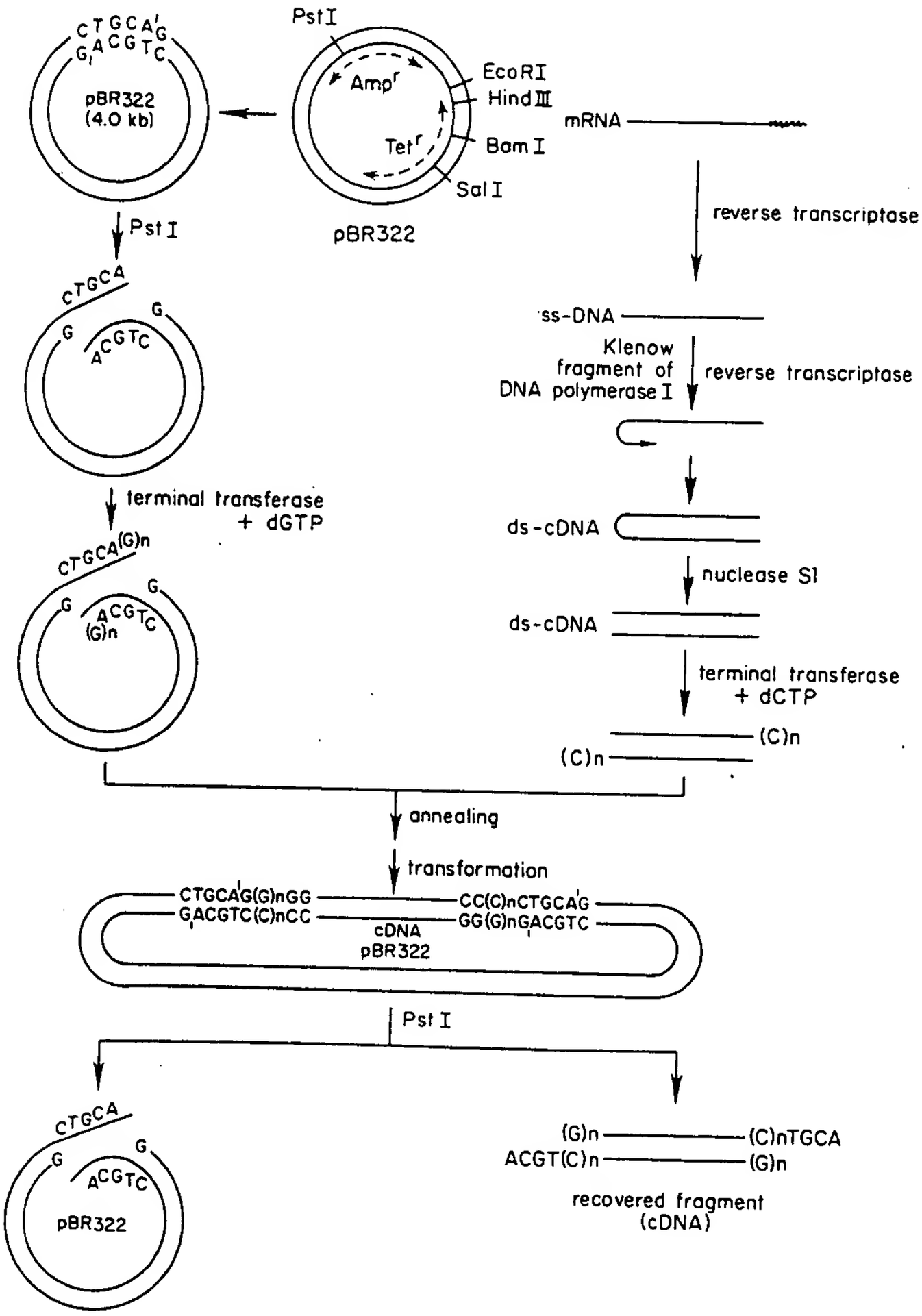


Figure 7.1

The conditions first used to achieve full-length, second-strand cDNA synthesis by DNA polymerase I (Efstratiadis et al. 1976) are still widely used (Wickens et al. 1978). In brief, the reaction is carried out at pH 6.9 to minimize the $5' - 3'$ exonuclease activity of DNA polymerase I and at 15°C to minimize the possibility of synthesizing "snapback" DNA. The Klenow fragment of DNA polymerase I, which lacks the $5' - 3'$ exonuclease activity, has also been successfully employed to synthesize the second cDNA strand.

Okayama and Berg find that full-length or nearly full length cDNA copies are preferentially converted to duplex cDNA, and an efficiency of approximately 100,000 transformants per microgram of starting mRNA is obtained. The preferential cloning of long cDNA transcripts is thought to be a consequence of the preferential utilization of full-length reverse transcription by terminal transferase. They speculate that shortened or truncated cDNA strands in the mRNA · DNA duplex are not efficiently recognized by the terminal transferase and are therefore selected against. Although the rabbit α- and β-globin mRNA was used to establish this cDNA cloning procedure, Okayama and Berg indicate that other cDNA clones representing both rare and long (6500-nucleotide) mRNAs have been obtained with this procedure.

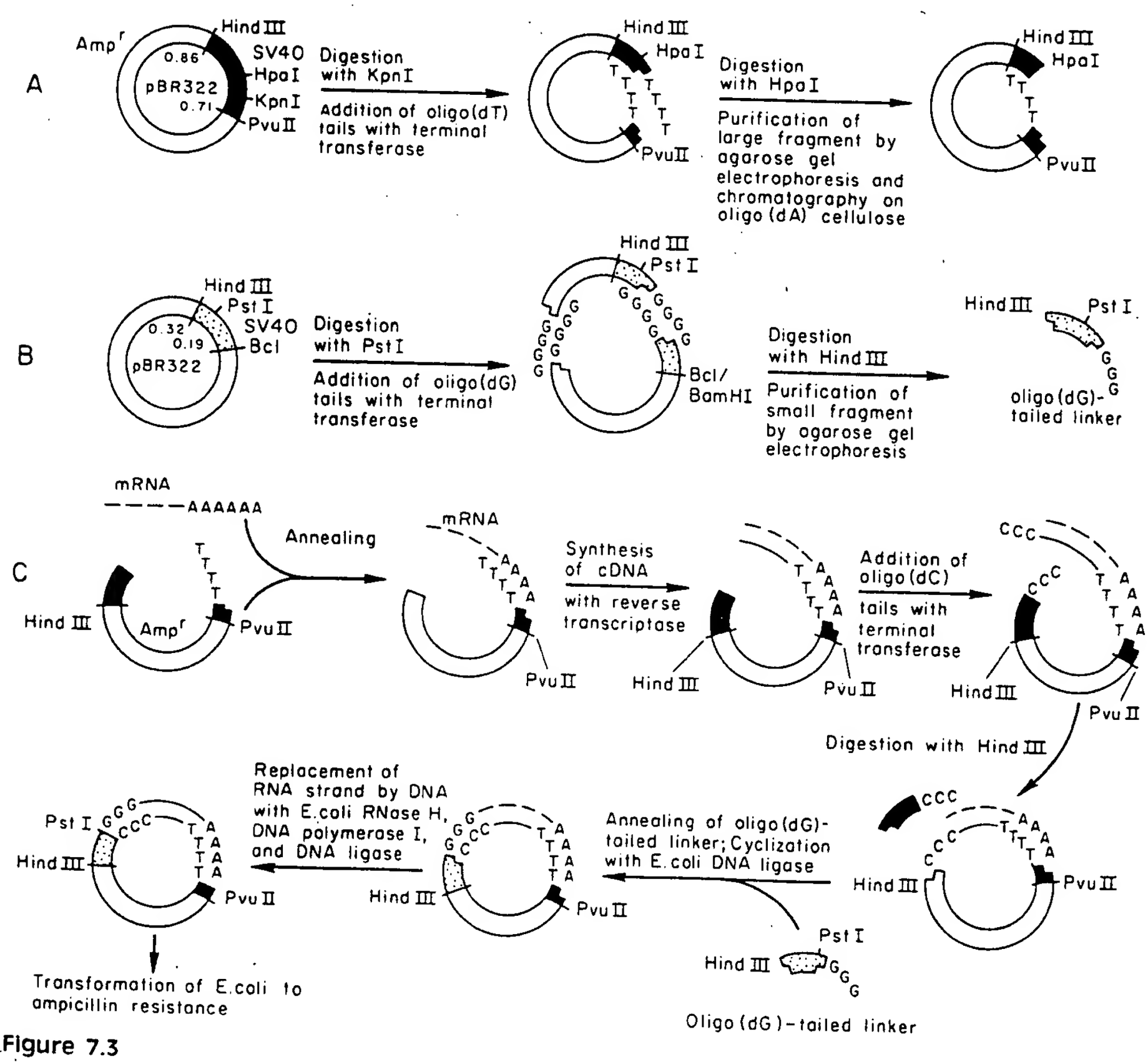


Figure 7.3

Preparation of (*A*) plasmid primer and (*B*) oligo(dG)-tailed linker DNA. (*C*) Steps in the construction of plasmid-cDNA recombinants. pBR322 DNA is represented by the open sections of each ring; SV40 DNA is indicated by the darkened or stippled segments. The numbers next to the restriction site designations are the corresponding SV40 DNA map coordinates.